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Experiments outlined in the original statement of work showed that our initial sustained release technology for the delivery of therapeutic adenovirus was not feasible. As a result, we have developed a revised statement of work that entails the development of a controlled release formulation of tumor necrosis factor α (TNFα) in combination with an adenovirus that replicates selectively within carcinoma tissue and sensitizes the tumor tissue to TNFα. We have succeeded in developing microspheres that release bioactive TNFα over more than 1 month. We have also succeeded in obtaining the selectively replicating adenovirus (*dl*338). We have also succeeded in demonstrating a greater than additive tumoricidal activity *in vitro* of the TNFα combined with the *dl*338. These experimental successes will be followed in the current year by animal experiments expected to validate this novel therapy for breast carcinoma.

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5. INTRODUCTION

The original grant proposal was directed towards the development of targetable microspheres that would release recombinant adenovirus over a sustained period of time within breast carcinoma tissue. This project is a collaborative effort between the Department of Biomedical Engineering at Johns Hopkins University (JHU) and the Department of Medicine at the University of Alabama at Birmingham (UAB). At the time of the grant proposal submission, we had developed a novel microsphere formulation that released adenovirus in a time-dependent fashion [1]. An important aspect of this original formulation was the finding that the microspheres could be lyophilized, thereby stabilizing the formulation. In subsequent experiments performed by both UAB and JHU, we found that it was difficult to reproduce the successful retention of adenoviral bioactivity following lyophilization. The reasons for this will be discussed in the subsequent sections, however, we felt this was a major impediment to the completion of our original aims.

We have redirected our efforts towards a different controlled release system that is used in conjunction with a therapeutic adenovirus, and this is reflected in a modified Statement of Work (attached). The new direction involves the development of a controlled release tumor necrosis factor alpha (TNFα) that will be used in combination with a conditionally replicative adenovirus containing an intact E1A transcriptional unit. The rationale for this new direction is based on several points as follows: (i) TNFα is toxic when administered systemically so that local, controlled release will increase the therapeutic index, (ii) cells expressing the adenovirus E1A proteins have been shown by multiple investigators to be sensitized to the toxic effects of TNF [2-7], hence using an E1A adenovirus with TNF should result in a combined toxicity, (iii) the E1A-containing adenovirus is similar to a virus shown to have a biological selectivity for replication in neoplastic tissues [8-11], even when systemically administered - thereby providing an element of biological targeting of breast carcinoma tissues.

6. BODY

Original statement of work, task #1:

a) microspheres made with varied percentages of gelatin, alginate and calcium - other variables examined included systematic changes in the temperature of gelatin, alginate - we also carefully examined influence of vortex speed on size and size variability of the spheres:

Result summary - Although we did identify conditions that resulted in consistently sized spheres, we found that lyophilization reduced bioactivity by 2-3 orders of magnitude. We subsequently tried different lyophilization buffers (varying glycerol concentration) without any improvement. We consulted several biotechnology companies, learning that the only means of consistently preserving adenoviral bioactivity with lyophilization required a proprietary process with expensive, gradual lyophilization equipment used in the pharmaceutical industry. This technical barrier was felt to be insurmountable within the budgetary constraints of this grant, hence we shifted

efforts into our new statement of work.

Revised statement of work, task #1:

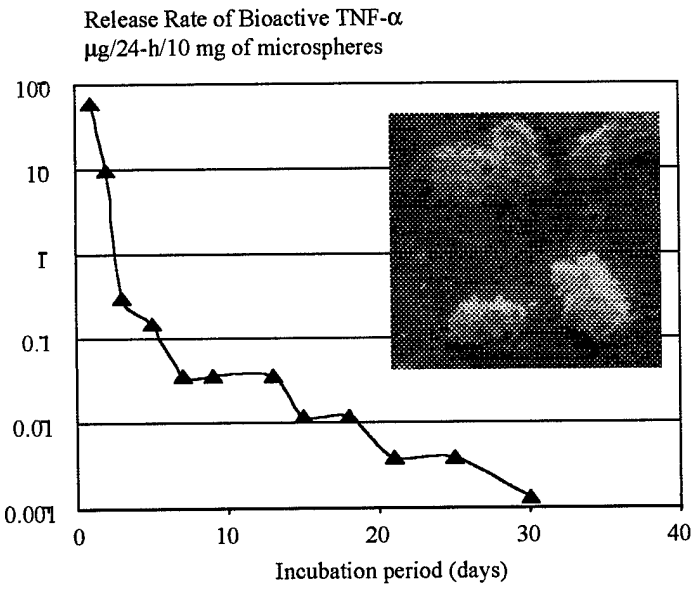
Synthesis of TNF-a Controlled Release Microspheres

Human serum albumin (HSA, 2.5%) was prepared from the injectable 25% HSA solution (Albumarc 25%, Baxter Healthcare Co., CA) and adjusted to pH 3.0. Heparin (1000 USP, Elkins-Sinn, NJ) purchased from the hospital pharmacy was used without any modification. TNF- (10^5U/ml) was added to the heparin solution before the coacervation. Microsphere formulation was achieved by adding HSA solution (3 ml) into a vortexing heparin solution (3 ml). After 10 sec of vortexing, the crosslinking reagent 1-ethyl 3 (3-propylamino) carbodiimide hydrochloride (EDC) was added to a final concentration of 3 mg/ml. After 15 min of reaction at RT, 0.1 M glycine (7ml) was added, and kept for another 15 min to quench the unreactive EDC. The typical encapsulation efficiency of TNF- was close to 95%.

In vitro Release studies

In vitro release studies were conducted by incubating the microspheres in 10% FCS medium at 37°C. The bioactivity of the released TNF- was assessed by determining the cytotoxicity of the cytokine on HGC-27 cells. Briefly, HGC-27 cells were seeded at a concentration of $5x10^4$ cells/well in 100 l culture medium containing actinomycin C_1 (1g/ml) and 100 l of the released medium into microtiter plates. After incubation for 24 h at 37°C and 5% CO_2, 10 l of cell proliferation reagent WST-1 was added to the wells and incubated for an additional 4 h. The absorbance of the wells ($A_{450nm} - A_{690nm}$) was measured and compared to a calibration curve to determine the bioactive concentration of TNF-.

The TNF- microparticles are irregular in shape, with a particle size range of 5-20 m. Release of TNF-from the microparticles follows a first-order kinetics and there is a burst in the first 24 h, although bioactive cytokine can still be detected for up to 3 weeks.

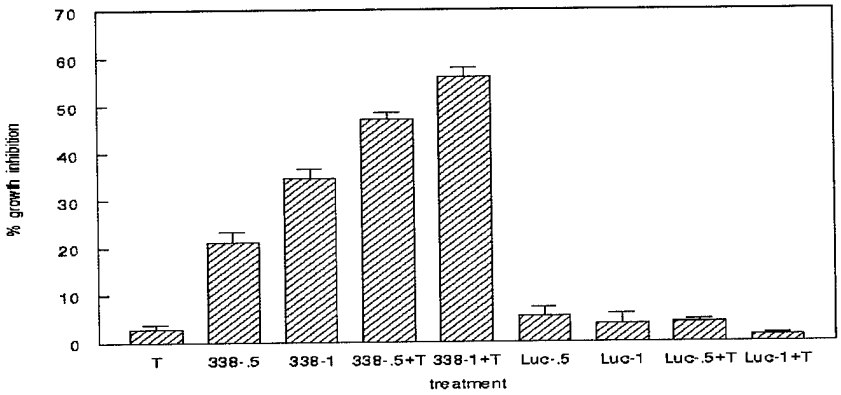


Task #2: Identify a conditionally replicative adenovirus suitable for use with the TNFα formulation

The objective here was the acquisition of an adenovirus that would efficiently transfer the E1A proteins into breast carcinoma tissue in order to sensitize the tissue to TNFα. The optimal virus would replicate selectively within the carcinoma tissue, and not in surrounding normal tissue so as to enhance the transfer of the adenoviral E1A within the tumor tissue. Using other funding sources, we had developed a virus (AdE1A-tk) that we had hoped would fulfill this objective. However, in pilot animal studies, we found that the virus did not replicate well within tumor nodules. As an alternative, we obtained the *dl*338 adenovirus for use in these studies. This virus contains a deletion within the E1B 55 kD -encoding region that greatly reduces its replication within normal tissues [12]. This virus is biologically similar to the Onyx-015 virus that has been developed as a tumor-specific oncolytic agent, and is now in Phase II clinical trials. We obtained a stock of this virus, and amplified it by standard methods. The identity of the virus was confirmed by selective sequencing of the E1B region.

Task #3: Evaluate individual and combined activity of the *dl*338 virus and TNFα *in vitro*

The *dl*338 virus and TNFα were used individually or in combination in the treatment of the lung carcinoma cell line, A549, *in vitro*. The results shown here are the mean of 3 experiments, and clearly demonstrated that the combination of virus and TNFα caused a significantly greater reduction in carcinoma cell growth than either of the treatments individually. For example, the TNF alone caused a 4% reduction, *dl*338 alone (moi=0.5) a 22% reduction, but the combination resulted in a 44% reduction.



Reduction of A549 growth by TNFα and/or adenovirus. Shown is the mean of 3 experiments ± S.E.M. Ordinate: % growth reduction determined by a colorimetric growth assay 5 days after treatment, abscissa: agents administered to the cells. "T"=TNFα 100 ng/ml, "338 "= *dl*338 with moi following hyphen, "Luc"=AdLuc, a control adenovirus lacking E1A region.

7. KEY RESEARCH ACCOMPLISHMENTS

- development of controlled release formulation of TNFα as a novel cancer therapeutic
- development of a novel therapeutic strategy for the treatment of carcinoma that employs sustained release TNF and adenovirus with an intact E1A region - feasibility demonstrated by *in vitro* experiments

8. REPORTABLE OUTCOMES

- invention disclosure filed with university for use of sustained release formulation of TNF

9. CONCLUSIONS

The original research plan has been revised in response to the unexpected technical difficulties encountered that called into question the ability to complete the subsequent tasks. A revised Statement of Work has been developed that incorporates similar themes as in the original proposal. This new directed is also highly novel, and employs the first described sustained release formulation of TNFα in combination with a selectively replicating adenovirus with anticipated biological selectivity for breast carcinoma tissue. In the next twelve months, we plan to continue progress along the revised Statement of Work with completion of the initial animal experiments.

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Statement of Work-Revised 10/99

1. Task #1: Construct and characterize microspheres that contain and release tumor necrosis factor α (TNFα) over an extended period of time
    a) identify a formulation that encapsulates TNFα in a bioactive form
    b) develop an assay that can measure small amounts of TNFα released from the encapsulated formulation
    c) modify the above formulation to release TNFα over a 10-30 day time period

2. Task #2: Identify a conditionally replicative adenovirus suitable for use in combination with the extended release TNFα formulation
    a) obtain *dl*338 virus
    b) amplify virus, confirm identity by limited sequence analysis of E1B region

3. Task #3: Evaluate individual and combined activity of *dl*338 virus and TNFα *in vitro*
    a) test *dl*338 and TNFα on lung carcinoma cell line *in vitro*
    b) test *dl*338 and TNFα on MCF7 breast carcinoma cell line *in vitro*

4. Task #4: Evaluate the combined activity of *dl*338 virus and TNFα *in vivo* by intratumoral injection
    a) administer the *dl*338 and TNFα by intratumoral injection to MCF7 tumor nodules
    b) assess distribution of virus in MCF7 tumor nodule by rescue cultures and PCR analysis

5. Task #5: Evaluate combined activity of *dl*338 virus administered systemically and TNFα administered by intratumoral injection
    a) administer the *dl*338 to tumor bearing mice by tail vein injection, and administer the TNFα formulation by intratumoral injection
    b) assess distribution of virus in MCF7 tumor nodule by rescue cultures and PCR analysis